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THE EFFECTS OF LOW LEVELS OF AFLATOXIN B1 ON HEALTH, GROWTH PERFORMANCE AND REPRODUCTIVITY IN MALE RABBITS

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Abstract: This study investigated the chronic effects of relatively low exposure to aflatoxin B1 (AFB1) on the growth performance, immune situation and reproduction in male rabbits. Bucks (n=32, 4.82±0.22 kg) were individually assigned to 4 treatments (8 replicates each) using a randomised complete block design. Four diets containing 0, 0.02, 0.05, and 0.1 mg AFB1/kg, were provided to bucks for 8 wk. Growth performance and semen quality were measured. Blood, organ and tissue samples were collected to measure haematological indices, liver function, organ weights and immune parameters. Compared to control, AFB1-contaminated diets reduced body weight and average daily gain ($P<0.05$), altered certain haematological indices and liver function with decreased monocytes percentage and mean corpuscular haemoglobin concentration, and increased plateletcrit and albumin ($P<0.05$), slightly impaired reproductive parameters with enhanced ratio of morphologically abnormal sperm cells at early stage and reduced post-stage acrosome integrity, testis weight and serum testosterone concentration ($P<0.05$), decreased immune function with reduced relative liver weight (%) and tumour necrosis factor-α levels in serum and liver tissue, and increased serum 8-hydroxy-2'-deoxyguanosine levels ($P<0.05$). Furthermore, bucks fed diets with relatively high AFB1 (0.05 and 0.1 mg AFB1/kg) had reduced red blood cell and haematocrit ($P<0.05$) in contrast with the low AFB1 group (0.02 mg AFB1/kg). In conclusion, diets containing 0.05 and 0.1 mg AFB1/kg had negative effects on bucks' growth performance, haematology, reproductivity and immune function, whereas diet containing 0.02 mg AFB1/kg had only minor effects on the parameters measured.

Key Words: rabbits, aflatoxin, growth performance, immune function, reproductivity .

INTRODUCTION

Mycotoxins are the secondary metabolites of fungi that are invisible but typically exist in feed even when the particular fungi are no longer present (Cotty and Jaime-Garcia, 2007). To date, more than 300 mycotoxins have been identified, and aflatoxins are the most common and toxic among them (Abdel-Wahab *et al.,* 2002). Aflatoxins are produced by several species of the fungus *Aspergillus*, including *A. parasiticus*, *A. numius*, and *A. pseudotamarii*, and the most common form is *A. flavus*. Aflatoxicosis can cause recurrent serious health problems and important economic losses (Peters and Teel, 2003; Gong *et al.,* 2004; Williams *et al.,* 2004; Abnet, 2007). Four aflatoxins were initially isolated and identified as aflatoxin B_1, B_2, G_1 and G_2, and aflatoxin B_1 (AFB1) was the most harmful (Eisa and Metwally, 2011). The growth of *Aspergillus* species and the production of aflatoxins require certain favourable environmental conditions, including high temperature and humidity (Ellis *et al.,* 1991). In addition, there is a second chance for contamination with aflatoxin under the above conditions during the storage time. Thus, aflatoxicosis is an extreme challenge in hot and humid regions. For instance, 77% of rabbit feed samples analysed in India were contaminated with aflatoxins (Mohanamba *et al.,* 2007), and the rate of contamination was above 50% in China (Fan *et al.,* 2012).

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Different animal species exhibit varied sensitivity to the toxic effects of AFB1. Among domestic animals, rabbits are highly susceptible to AFB1 (Salem *et al.,* 2001). Guerre *et al.* (1996) reported a significant decrease in body weight (BW) and the activity of drug metabolising enzymes in rabbits with the oral administration of 0.1 mg/kg AFB1. Eisa and Metwally (2011) reported thrombocytopenia and hepatotoxicity in rabbits fed a ration naturally contaminated with AFB1 (0.1 mg/kg ration). AFB1 has negative effects on sperm quality in species, such as rats, mice, sheep and pigs (Isaac *et al.,* 2014). In addition, previous studies showed that AFB1 suppressed testosterone (T) secretion by disrupting the androgen biosynthetic pathway in rat Leydig cells (Hancock *et al.,* 2009; Sherrill *et al.,* 2010). Similar results were observed in mice, pigs and sheep (Abdelaziz *et al.,* 2009; Ewuola *et al.,* 2014). However, limited studies focus on the reproductive toxicity of AFB1 in rabbits. Therefore, the aim of the present study was to investigate the effects of relatively low levels of AFB1 on the health, growth performance and reproduction of male rabbits.

MATERIALS AND METHODS

The protocol for the use of animals in this study was approved by the Southwest University Animal Care and Use Committee.

Animals and experimental design

The experiment was performed on the experimental rabbit farm of Southwest University (Chongqing, China). Thirty-two IRA male rabbits were purchased from Axingji Rabbit Company (Chongqing, China) at 7 mo of age. Bucks were housed in a naturally ventilated building and individually housed in wire-net cages (80×60×50 cm), provided with free water but fixed and limited feed in order to maintain high semen quality and avoid feed waste (180 g/d, based on the feed intake experienced during adaption period). After a 5-d adaption period, 32 bucks were randomly distributed into 4 groups (8 replicates each) with different levels of aflatoxin. The experiment lasted 8 wk based on the rabbit's sperm production cycle, which allowed sufficient time for the AFB1 to accumulate in tissues (Morton, 1988). Bucks in the control group were fed a commercial diet from Axingji Rabbit Company, whereas bucks of the 3 treated groups were given control diet mixed with 0.02, 0.05, and 0.1 mg AFB1/kg. Purified AFB1 from *Aspergillus flavus* was purchased from Sigma (St. Louis, Mo, USA). The pelleted commercial rabbit diets were first ground into powder, and purified AFB1 were gradually diluted by mixing with feed powder to meet the target concentration and quantity, then feed powders with different level of AFB1 were mixed and pelleted individually before feeding.

Sampling

Bucks were weighed every week. Data were recorded before feeding in the morning, and then the average daily gain (ADG) was calculated. During the adaption period, bucks were trained for semen collection using an artificial vagina. Thereafter, ejaculates were collected biweekly to measure semen quality. At the end of the feeding period, blood samples were obtained by means of cardiac puncture from all bucks before euthanisation. For each buck, blood samples were collected in different vacutainers (BD, Franklin Lakes, NJ). Vacutainers containing EDTA (7 mL) were used to obtain whole blood samples for haematological measurements, whereas vacutainers without anticoagulant (10 mL) were used to collect serum for liver function, tumour necrosis factor-α (TNF-α), malondialdehyde (MDA), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) tests. After euthanisation, the intestinal tract was quickly removed, and the small intestine was dissected. The middle section of the jejunum was isolated, flushed with distilled water and then opened for scraping the mucosal layer. The weights of liver, gallbladder, spleen, kidney, testis and epididymis were measured, and these weights as a percentage of BW were also calculated. Mucosa and liver samples were homogenised in phosphate buffer (pH 7.4) and centrifuged (1700 rpm at 4°C) for 10 min, then the supernatant was stored at –80°C until MDA, TNF-α and 8-OHdG concentration analysis.

Semen evaluation

Sperm motility was immediately measured using an Olympus inverted microscope (100×magnification). Ten µL of collected semen was placed directly onto a microscope slide maintained at 37°C and covered by a coverslip. Sperm motility estimations were expressed from 0 to 1 and were performed in 3 different microscope fields to obtain the mean motility score for each semen sample. An additional 25 µL of collected semen was diluted (1:80) with 5%

glucose solution. Sperm concentrations were determined using a haemocytometer with diluted semen. Sperm cells in the 5 squares of the ruled area were counted diagonally. Then, the average of 2 counts was multiplied by the dilution factor and number of squares, and the volume was converted to mL as the final sperm concentration. The percentage of morphologically abnormal sperm cells and acrosomal integrity were measured using Coomassie brilliant blue staining and examined with an Olympus inverted microscope (400×magnification). Abnormal morphology of sperms included tail defects, detached heads, and abnormal mid-pieces (Ataman *et al.,* 2014). The remaining semen samples were centrifuged (1500 rpm) to obtain the supernatants and subsequently used for the detection of pH values and osmotic pressures. Osmotic pressure was measured using a freezing point osmometer, and the unit was expressed as mOsm (mOsmol/kg).

Haematological measurements and liver function test

White blood cells, lymphocytes, monocytes, neutrophils, lymphocytes percentage, monocytes percentage, neutrophils percentage, red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT%), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT%) in whole blood were determined as haematological indicators using an auto haematology analyser (BC-2800Vet, Mindray, China). The serum concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK phosphatase), prealbumin (PAB), total protein, albumin, globulin, albumin to globulin ratio (A/G ratio) and γ-glutamyltransferase (γ-GT) were tested for liver function using an automated chemistry-immuno analyser (7600-010, Hitachi, Japan).

Serum testosterone

Testosterone (T) in serum was measured using a Rabbit T ELISA Kit (DongGe, Inc., Beijing, China) as an indicator of endocrine changes as a result of aflatoxicosis in male reproductive processes. Briefly, 10 µL of samples plus diluent or 50 µL of standards were added to microplate wells that were previously coated with a capture antibody in conjunction with biotinylated antibody reagent. Detection was performed using horse radish peroxidase (HRP), chromogen solution A and B, and a stop solution of 0.18 M H_2SO_4. Absorbance was read at 450 nm by an ELISA plate reader (Bio-rad, USA).

Serum and tissue TNF-α, MDA and 8-OHdG

Jejunum mucosa was homogenised in phosphate buffer saline (PBS) containing protease inhibitors, and the supernatant was collected and analysed for protein content using a bicinchoninic acid (BCA) assay. The TNF-α, MDA and 8-OHdG in the supernatant and serum were measured using Rabbit ELISA Kits (DongGe, Inc., Beijing, China) with the same instructions as the Rabbit T ELISA Kit.

Statistical analysis

This study used a randomised complete block design, and the initial BW was the block. One buck in each cage was considered as an experimental unit. Data were analysed using the MIXED procedures in SAS software (SAS Inst. Inc., Cary, NC). Probability values less than 0.05 were considered statistically significant.

RESULTS

Growth performance

The initial BW of the bucks did not differ among treatments (Table 1). During the first 3 wk and week 7, the supplementation of AFB1 did not affect bucks' BW. At week 4, the BW of the control bucks was significantly higher ($P<0.05$) compared to the bucks fed 0.1 mg AFB1/kg. At week 5, the BW of the bucks fed 0.02 mg AFB1/kg was significantly higher ($P<0.05$) compared to those fed 0.1 mg AFB1/kg. At week 6, the BW of the bucks fed 0.02 mg AFB1/kg was significantly higher ($P<0.05$) than the bucks fed 0.05 and 0.1 mg AFB1/kg, separately. At

Table 1: Effects of aflatoxin B1 (AFB1) on growth performance of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
BW (kg)					
initial BW	4.82	4.82	4.82	4.83	0.081
wk1 BW	4.84	4.85	4.87	4.78	0.086
wk2 BW	4.89	4.86	4.85	4.83	0.088
wk3 BW	5.00	4.98	4.95	4.90	0.088
wk4 BW	5.07^{b}	5.04^{ab}	5.00^{ab}	4.94^{a}	0.085
wk5 BW	5.07^{ab}	5.11^{b}	5.02^{ab}	4.99^{a}	0.092
wk6 BW	5.07^{ab}	5.12^{b}	4.98^{a}	4.99^{a}	0.087
wk7 BW	5.14	5.18	5.04	5.03	0.108
wk8 BW	5.12^{bc}	5.15^{c}	4.98^{ab}	4.96^{a}	0.089
ADG (g)					
wk 1	3.39	4.29	7.68	–2.04	4.225
wk 2	6.43	2.32	–3.75	2.13	3.345
wk 3	15.54	15.18	18.37	10.36	2.639
wk 4	9.38	8.91	6.56	7.61	1.539
wk 5	–0.18	6.77	2.14	6.25	2.742
wk 6	0.42	1.67	–0.95	0.63	2.604
wk 7	8.91	7.03	12.45	5.00	2.926
wk 8	–3.75	–4.58	–10.00	–12.08	5.431
overall	5.40^{bc}	5.91^{c}	2.81^{ab}	2.28^{a}	1.085

BW: body weight; ADG: average daily gain. SEM: standard error mean.

^{abc}Means in the same row having different superscripts are significantly different ($P<0.05$).

the end of the study, the BW of the control bucks was significantly increased ($P<0.05$) compared to the bucks fed 0.1 mg AFB1/kg, whereas the BW of the bucks fed 0.02 mg AFB1/kg was significantly higher ($P<0.05$) compared to the bucks fed 0.05 and 0.1 mg AFB1/kg, separately.

The ADG of bucks from week 1 to week 8 did not differ among treatments. However, the overall ADG of the control bucks was significantly more ($P<0.05$) compared to the bucks fed 0.1 mg AFB1/kg, and the overall ADG of the bucks fed 0.02 mg AFB1/kg was significantly higher ($P<0.05$) compared to the bucks fed 0.05 and 0.1 mg AFB1/kg, separately. No significant difference in feed intake was observed as all animals consumed the ration of 180 g feed/animal without any left-over.

Haematological measurements and liver function

The percentage of monocytes in bucks fed 0.02 mg AFB1/kg was significantly decreased ($P<0.05$) compared to the bucks in control (Table 2). The red blood cells and percentage of haematocrit from the bucks fed 0.02 mg AFB1/kg were significantly more ($P<0.05$) than the bucks fed 0.05 and 0.1 mg AFB1/kg, separately. The bucks fed 0.02 mg AFB1/kg exhibited higher MCHC ($P<0.05$) compared to the bucks fed 0.05 and 0.1 mg AFB1/kg, separately.

Albumin levels were significantly increased ($P<0.05$) in the bucks fed 0.1 mg AFB1/kg compared to the other groups (Table 3).

Semen quality and testosterone concentration

The supplementation of AFB1 did not affect bucks' sperm motility, concentration, pH value or osmotic pressure from weeks 2 to 8 (Table 4). At week 2, the percentage of cells with abnormal morphology from the bucks fed 0.1 mg AFB1/kg was significantly higher ($P<0.05$) compared to the controls and bucks fed 0.05 mg AFB1/kg. At week 4, the acrosomal integrity of the bucks fed 0.05 mg AFB1/kg was significantly reduced ($P<0.05$) compared to

Table 2: Effects of aflatoxin B1 (AFB1) on haematological measurements of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
White blood cells (10^9/L)	5.33	6.85	6.98	6.43	0.747
Lymphocytes (10^9/L)	2.86	3.63	3.69	3.76	0.367
Monocytes (10^9/L)	0.18	0.14	0.16	0.15	0.023
Neutrophils (10^9/L)	2.33	2.90	2.28	2.51	0.430
Lymphocytes %	54.25	54.53	57.35	59.90	4.792
Monocytes %	3.05^{b}	2.38^{a}	2.48^{ab}	2.50^{ab}	0.188
Neutrophils %	42.13	41.38	34.60	37.60	3.663
RBC (10^{12}/L)	5.598^{ab}	5.842^{b}	5.433^{a}	5.569^{a}	0.108
HGB (g/L)	118.5	121.8	115.8	117.6	2.509
HCT%	37.83^{ab}	39.34^{b}	37.10^{a}	37.28^{a}	0.665
MCV (fL)	66.46	68.16	68.4	67.13	0.801
MCH (pg)	21.13	21.08	21.25	21.09	0.287
MCHC (g/L)	318.9^{b}	309.9^{a}	311.5^{a}	315.10^{ab}	2.183
RDW%	13.71	13.96	13.48	13.81	0.296
PLT (10^9/L)	306.1	321.1	349.5	376.0	52.714
MPV(fL)	5.73	5.61	5.63	5.59	0.134
PDW	16.52	16.48	16.43	16.36	0.103
PCT%	0.171^{a}	0.220^{ab}	0.212^{ab}	0.227^{b}	0.020

C: control diet; T1: control diet with 0.02 mg AFB1/kg; T2: control diet with 0.05 mg AFB1/kg; T3: control diet with 0.1 mg AFB1/kg, n=8/treatment. SEM: standard error mean. RBC: red blood cell; HGB: haemoglobin; HCT%: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; RDW: red blood cell distribution width; PLT: platelet count; MPV: mean platelet volume; PDW: platelet distribution width; PCT%: plateletcrit.
^{ab}Means in the same row having different superscripts are significantly different (P<0.05).

the controls. At week 8, the acrosomal integrity of the bucks fed 0.1 mg AFB1/kg was significantly lower (P<0.05) compared to other groups.

Serum testosterone concentrations in the bucks fed 0.1 mg AFB1/kg were significantly reduced (P<0.05) compared to the control bucks and bucks fed 0.02 mg AFB1/kg.

Table 3: Effects of aflatoxin B1 (AFB1) on liver function of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
ALT (U/L)	79.00	86.63	83.75	95.63	10.499
AST (U/L)	56.50	63.25	59.46	59.00	9.340
ALK (U/L)	36.29	33.13	32.00	30.75	3.243
PAB (mg/L)	178.76	175.79	170.41	185.69	9.858
TP (g/L)	58.49	59.35	59.93	62.56	1.501
Albumin (g/L)	40.04^{a}	40.44^{a}	39.87^{a}	41.7^{b}	0.470
Globulin (g/L)	19.35	18.93	20.05	20.86	0.933
A/G ratio	2.06	2.14	2.01	2.02	0.100
γ-GT (IU/L)	7.63	5.38	7.50	7.69	0.988

C: control diet; T1: control diet with 0.02 mg AFB1/kg; T2: control diet with 0.05 mg AFB1/kg; T3: control diet with 0.1 mg AFB1/kg, n=8/treatment. SEM: standard error mean. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALK: alkaline phosphatase; PAB: prealbumin; TP: total protein; A/G ratio: albumin to globulin ratio; γ-GT: γ-glutamyltransferase.
^{ab}Means in the same row having different superscripts are significantly different (P<0.05).

Table 4: Effects of aflatoxin B1 (AFB1) on semen quality and testosterone concentration of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
Sperm motility					
wk2	0.83	0.87	0.91	0.91	0.046
wk4	0.81	0.85	0.90	0.74	0.071
wk6	0.94	0.97	0.90	0.94	0.045
wk8	0.84	0.82	0.87	0.80	0.069
Sperm Concentration (10^8/mL)					
wk2	4.120	6.062	5.121	4.623	0.742
wk4	5.178	5.982	6.042	5.204	0.580
wk6	4.885	4.239	6.593	4.860	1.007
wk8	4.277	4.230	5.555	5.671	0.743
Abnormal sperm (%)					
wk2	23.2^{a}	27.8^{ab}	24.8^{a}	36.3^{b}	3.624
wk4	38.9	24.6	26.1	31.9	6.524
wk6	42.3	35.3	37.4	36.2	3.265
wk8	32.7	25.3	31.6	30.6	5.404
Acrosomal integrity (%)					
wk2	97.0	98.6	98.4	97.6	0.446
wk4	99.7^{b}	99.4^{ab}	99.2^{a}	99.4^{ab}	0.161
wk6	93.1	93.2	93.3	93.2	0.112
wk8	94.2^{b}	93.9^{b}	94.5^{b}	89.9^{a}	1.171
pH value					
wk2	7.11	6.97	7.02	7.01	0.113
wk4	6.98	7.04	6.93	7.01	0.103
wk6	6.86	6.80	6.75	6.78	0.112
wk8	6.80	6.64	6.61	6.64	0.085
Osmotic pressures (mOsm)					
wk2	253.8	254.8	256.2	253.3	7.695
wk4	199.6	235.1	225.5	201.9	15.042
wk6	245.8	234.9	223.1	220.1	15.107
wk8	244.2	232.8	235.1	219.5	16.565
Serum testosterone (pg/mL)	64.613^{b}	64.906^{b}	53.944^{ab}	47.074^{a}	4.485

C: control diet; T1: control diet with 0.02 mg AFB1/kg; T2: control diet with 0.05 mg AFB1/kg; T3: control diet with 0.1 mg AFB1/kg, n=8/treatment and sampling point. SEM: standard error mean. WK: week (in Table 1 it was 'wk' – be consequent).
^{ab}Means in the same row having different superscripts are significantly different (P<0.05).

Organ weights

There was no effect of AFB1 on the weights and relative organ weight (%) of gall bladder, spleen, kidney and epididymis (Table 5). The control bucks had higher liver weights (P<0.05) compared to those fed 0.05 and 0.1 mg AFB1/kg, and liver weights as a percentage of BW in the controls were also greater (P<0.05) than in AFB1 treated animals. The control bucks exhibited increased testis weight (P<0.05) compared to those fed 0.1 mg AFB1/kg, however the differences in the relative weights were not significant.

Cytokine

In the serum, the control bucks and bucks fed 0.02 mg AFB1/kg had increased TNF-α concentrations (P<0.05) compared to the bucks fed 0.1 mg AFB1/kg (Table 6). The control bucks and bucks fed 0.02 mg AFB1/kg exhibited rising TNF-α concentrations (P<0.05) in liver compared to the bucks fed 0.05 and 0.1 mg AFB1/kg, separately. In the jejunum, the control bucks had lower TNF-α concentrations (P<0.05) in contrast with the bucks fed 0.05 and

Table 5: Effects of aflatoxin B1 (AFB1) on absolute and relative organ weights of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
Organ weight (g)					
Liver	138.78[b]	130.56[ab]	121.60[a]	124.66[a]	4.265
Gallbladder	1.43	1.25	1.09	1.12	0.218
Spleen	1.49	1.35	1.46	1.46	0.128
Kidney	19.75	20.45	19.99	20.09	0.548
Testis	4.29[b]	3.73[ab]	3.80[ab]	3.61[a]	0.225
Epididymis	1.45	1.59	1.54	1.48	0.056
Relative organ weight (%)[1]					
Liver	2.70[b]	2.52[a]	2.45[a]	2.51[a]	0.055
Gallbladder	0.026	0.025	0.022	0.021	0.004
Spleen	0.029	0.028	0.030	0.029	0.003
Kidney	0.384	0.398	0.401	0.406	0.011
Testis	0.084	0.074	0.076	0.074	0.005
Epididymis	0.030	0.033	0.031	0.030	0.001

[1]Expressed as organ weight (g)/body weight (g); C: control diet; T1: control diet with 0.02 mg AFB1/kg; T2: control diet with 0.05 mg AFB1/kg; T3: control diet with 0.1 mg AFB1/kg, n=8/treatment. SEM: standard error mean.
[ab]Means in the same row having different superscripts are significantly different ($P<0.05$).

0.1 mg AFB1/kg, separately, whereas the bucks fed 0.02 mg AFB1/kg exhibited reduced TNF-α concentrations ($P<0.05$) compared to the bucks fed 0.05 mg AFB1/kg.

Oxidative status

In the liver, AFB1 did not affect the MDA concentrations. The bucks fed 0.02 mg AFB1/kg had reduced serum MDA concentration ($P<0.05$) compared to the control bucks and bucks fed 0.05 mg AFB1/kg, separately. The control bucks and bucks fed 0.02 mg AFB1/kg had lower MDA concentrations ($P<0.05$) in the jejunum compared to the bucks fed 0.05 and0.1 mg AFB1/kg, separately.

Table 6: Effects of aflatoxin B1 on cytokine, oxidative status and DNA damage in the serum, jejunum and liver of bucks (n=8/treatment).

	AFB1 level (mg/kg)				
	0	0.02	0.05	0.1	SEM
TNF-α					
Serum (ng/L)	73.32[b]	71.24[b]	62.14[ab]	55.10[a]	4.913
Jejunum (ng/g)	149.08[a]	170.70[ab]	265.49[c]	263.97[bc]	31.706
Liver (ng/g)	377.01[b]	386.58[b]	269.18[a]	292.38[ab]	53.390
MDA					
Serum (µmoL/L)	2.51[b]	1.78[a]	2.48[b]	2.06[ab]	0.221
Jejunum (µmoL/g)	4.14[a]	4.42[a]	8.67[b]	7.83[b]	0.623
Liver (µmoL/g)	3.40	3.33	3.92	3.93	0.368
8-OHdG					
Serum (µg/L)	12.59[a]	13.12[ab]	15.58[b]	13.44[ab]	1.104
Jejunum (µg/g)	37.83	39.74	38.16	31.77	3.543
Liver (µg/g)	16.07	18.29	14.96	15.34	1.149

C: control diet; T1: control diet with 0.02 mg AFB1/kg; T2: control diet with 0.05 mg AFB1/kg; T3: control diet with 0.1 mg AFB1/kg, n=8/treatment. SEM: standard error mean. TNF-α: tumour necrosis factor-α; MDA: malondialdehyde; 8-OHdG: 8-hydroxy-2 deoxyguanosine.
[abc]Means in the same row having different superscripts are significantly different ($P<0.05$).

DNA damage

In jejunum and liver, AFB1 did not affect 8-OHdG concentration. Control bucks exhibited reduced 8-OhdG concentrations ($P<0.05$) in serum compared to bucks fed 0.05 mg AFB1/kg.

DISCUSSION

Toxic effect of major mycotoxins on rabbits have been summarised by Mézes and Balogh (2009). However, for aflatoxicosis, morbidity, mortality, mean lethal dose, haemolytic anaemia, and liver lesions were observed only under high levels of aflatoxin. Reports on the chronic effects of low aflatoxin exposure on growth performance, haematology, spermatology and organ traits in rabbit are scarce. In rabbit, the short-term oral AFB1 dose that caused reductions in body weight and liver metabolising enzyme activities was 0.1 mg/kg, whereas the experimental period lasted only 5 d (Guerre *et al.,* 1996). Before that, Richard and Thurston (1975) reported growth retardation and reduced phagocytic activity in rabbits after 2-wk supplementation of AFB1 at concentrations greater than 0.03 mg/kg. Regarding the chronic effects of aflatoxicosis, oral treatments with 15 and 30 µg/kg BW for 9 wk reduced live body weight (LBW) and sperm quality (Salem *et al.,* 2001). Additionally, the previous studies providing 0.5 mg AFB1/kg or 0.833 mg AF/kg (including 0.135 mg AFB1/kg) through contaminated feed reported negative effects on growth performance, relative organ weight, histopathological status and antioxidant enzyme activities (Prabu *et al.,* 2013; Soliman *et al.,* 2001). As one of the most sensitive species to aflatoxin, our study aimed to investigate effects of chronic and low level of aflatoxin load on the growth performance, haematology and spermatology in male rabbits.

In this study, the bucks did not differ significantly in BW among treatments in the first 3 wk, but the bucks fed AFB1 had decreased BW from weeks 4 to 8. This result was similar to those of a previous study indicating that 0.5 mg AFB1/kg did not affect BW during the first 15 d, and then BW decreased from day 16 to 60 (Prabu *et al.,* 2013). In swine and broiler chicken, previous studies indicated that prolonged exposure to low levels of aflatoxin had negative effects on growth performance in the late feeding period (Marin *et al.,* 2002; Fan *et al.,* 2013; Gholami-Ahangaran and Zia-Jahromi, 2013; Rustemeyer *et al.,* 2014). The adverse effects of aflatoxin on animals' growth performances might be due to anorexia, reluctance to eat, inhibition of protein, DNA and RNA synthesis, and lipogenesis (Yu, 1982; Oguz and Kurtoglu, 2000). As bucks were fed fixed and limited diets without feed waste records, reduced growth performances were mainly related to lower nutrients utilisation. The length of exposure to aflatoxin and the contaminated level in the diet could affect the animal's response in terms of growth performance (Yunus *et al.,* 2011).

Feeding bucks with high level of AFB1 resulted in significant reduction of RBC, HCT% and MCHC compared to bucks fed a low level of AFB1 or the control group. Similar results were observed in rabbits, broilers and swine (Eisa and Metwally, 2011; Kecici *et al.,* 1995; Weaver *et al.,* 2013). Reduced RBC indicated the anaemic response to aflatoxicosis, which may be due to the reduced serum iron and altered protein metabolism. HCT% was measured as the concentration of RBC in a certain volume of blood, and it followed the same trends as RBC in this study. MCH concentration was important for the assortment of anaemia and RBC disorders, which were consistent with the RBC results. The slight monocytopaenia observed in this study could be explained by the inhibition of protein synthesis and cell proliferation induced by AFB1. Increased albumin levels in the high AFB1 group may be attributed to dehydration (Kaneko *et al.,* 1997).

Reproductive efficiency is considered the most crucial and restrictive factor in animal production, and high-quality semen production with well-developed reproductive organs is crucial to guarantee this efficiency. Spermatogenesis occurs in the seminiferous tubules of testes, and Sertoli cells located in the wall of these tubules stimulate the production and maturation of spermatozoa (Kärenlampi, 1987). In general, the above cells and tissues are protected by the blood-testis barrier, but AFB1 has the ability to pass the barrier and threaten spermatogenesis (Egbunike, 1982). In this study, the highest level of aflatoxin (0.1 mg AFB1/kg) in the diet increased the percentage of abnormal sperm at the beginning of the study and slightly decreased the acrosomal integrity during the chronic aflatoxicosis period. Previous studies have also reported increased rates of abnormal spermatozoa and acrosomal disorders using bulls, rams and broiler flocks suffering from chronic aflatoxicosis (Alm *et al*., 2002; Manafi *et al*., 2002; Yegani *et al*., 2006; Ataman *et al*., 2014). In addition, the procedure of sperm gaining fertility is dominated by the composition of internal epididymal conditions, which are directly regulated by testosterone. Adedara *et al.* (2014) demonstrated

that AFB1 disrupted the testosterone biosynthetic pathway in rat Leydig cells and suppressed testosterone secretion. The reduction of the serum testosterone level observed in this study may lead to a deteriorated internal epididymal milieu, which could also explain the changes in sperm morphology. Moreover, the impaired bioactivity and production of testosterone that relate to the degeneration of germinal epithelium may further negatively impact buck's testes weight (Faridha *et al.,* 2006; Verma and Mathuria, 2010). Thus, the bioactivity of testosterone could influence the morphology and functional integrity of testes and subsequently affect sperm production. In this study, we did not observe significantly reduced relative testis weight (%) in AFB1 treated bucks, which may be explained as low level of AFB1 consumption while bucks treated with sub-lethal doses of AFB1 showed significantly reduced relative testis weight (%) (Salem *et al.,* 2001).

Liver weight and relative liver weight (%) were often measured in terms of immune response to aflatoxicosis. Previous studies reported relatively high levels of AFB1 in animal diets of different species and observed increased liver weight and relative liver weight as a percentage of body weight, which may be due to specific and enlarged fatty liver induced by the inhibition of lipid transport (Tung *et al.,* 1972; Clarke *et al.,* 1986; Weaver *et al.,* 2013; Rustemeyer *et al.,* 2014). However, in our study, bucks fed diets with relatively low levels of AFB1 had decreased liver weights and liver weights as a percentage of BW. These results were similar to those of another study where broilers were fed different levels of AFB1 and AFB2 contaminated diets. The group with 25% naturally contaminated corn (containing 0.23 mg AFB1/kg) exhibited reduced liver weight and liver weight as a percentage of body weight compared to the control and groups with 50 to 100% naturally contaminated corn (Yang *et al.,* 2012). The mechanism was unclear and further studies are needed to test histopathology and relative immune gene expression.

Aflatoxin-induced oxidative damage results in an impaired immune system and tissue damage, including systemic inflammation, lipid peroxidation and DNA damage. Tumour necrosis factor-α values were commonly used as a measure of systemic inflammation, and the secretion of this inflammatory cytokine was inhibited by the anti-inflammatory cytokine IL-10, which was up-regulated by AFB1 contamination (Waal Malefyt *et al.,* 1991). In this study, the decreased TNF-α observed in the serum and liver may correlate with the down-regulation of inflammatory cytokine IL-10. Previous studies showed a numeric decrease in TNF-α mRNA synthesis and serum TNF-α concentration in pigs fed a low AFB1-contaminated diet, but less evidence has been documented in rabbits (Marin *et al.,* 2002; Weaver *et al.,* 2013). On the other hand, opposite results of TNF-α levels presented in jejunum where aflatoxins were mainly absorbed. Further studies were needed to explore the gene expression of IL-10 and TNF-α, and their relationship in jejunum. Malondialdehyde values were generally measured to describe cellular lipid damage caused by aflatoxin, and bucks fed 0.05 and 0.1 mg AFB1/kg exhibited increased MDA values in the jejunum. This result was consistent with a previous study using similar AFB1 levels in rabbit diets that indicated marked effects on increasing free radical occurrence in biological systems with low levels of AFB1 (Guerre *et al.,* 1999; Dönmez and Keskin, 2008). 8-Hydroxy-2'-deoxyguanosine was one of the most massive and highly mutagenic DNA lesions (David *et al.,* 2007). Serum 8-OHdG values increased due to the influence of AFB1 in current study, and this finding was supported by previous studies that determined serum 8-OHdG values in AFB1-treated rats (Asare *et al.,* 2007; Guindon-Kezis *et al.,* 2014).

CONCLUSIONS

Summing up data, bucks chronically consuming diets contaminated with 0.05 and 0.1 mg AFB1/kg exhibited restrained growth performance, modifications in haematological parameters, and impaired reproduction and immune function. Bucks fed diet contaminated with 0.02 mg AFB1/kg did not affect reproductive parameters, but slightly influenced haematology and relative liver weight (%).

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